# A Midgut Barrier to Tomato Spotted Wilt Virus Acquisition by Adult Western Flower Thrips

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This research was supported in part by the United States Department of Agriculture under Cooperative State Research Service special grant 88-34135-3593 managed by the Pacific Basin Advisory Group; Biomedical Research Support Grant, University of Hawaii-Manoa; and Research Centers in Minority Institutions grant RR-03061, National Institutes of Health.

We thank Shani Nakanishi, Maria Sumabat, Ronnie Natawidjaja, David Fisher, and Marilyn Dunlap for technical assistance and for the facilities that they provided. Special thanks to Dennis Gonsalves for providing TSWV antibodies and Min Wang for providing preparations of purified TSWV.

HITAHR journal series 3654.

Accepted for publication 8 June 1992.

## ABSTRACT

Ullman, D. E., Cho, J. J., Mau, R. F. L., Westcot, D. M., and Custer, D. M. 1992. A midgut barrier to tomato spotted wilt virus acquisition by adult western flower thrips. Phytopathology 82:1333-1342.

Ultrastructural and serological analyses provide evidence that a midgut barrier underlies the inability of adult western flower thrips (WFT), *Frankliniella occidentalis*, to acquire tomato spotted wilt virus (TSWV) through adult feeding on infected plants. As demonstrated for other thrips vector species, only WFT that acquire TSWV as larvae can successfully inoculate plants with the virus as adults. Transmission electron microscopy (TEM) observations and serological results demonstrate that larval and adult WFT ingest TSWV particles during feeding on infected plants, after which virions can be found within the midgut lumen and epithelial cells. Among adult thrips that ingest TSWV as adults and not as larvae, TSWV particles accumulate within masses of amorphous electron-dense material in the cytoplasm of midgut epithelial cells, frequently lack a visible surrounding membrane, and are sometimes associated with multivesicular bodies and rough endoplasmic reticulum. Dissemination of virus particles beyond the midgut epithelia could not be detected serologically or with TEM observation in adult thrips given acquisition feeding as adults. Serological evidence and TEM observations also show that although TSWV is ingested by adult WFT, it is not retained. In contrast, among larval thrips given acquisition feeding on virus-infected plants, TSWV was detected serologically and with TEM observation in the midgut epithelia and hemocoel. Furthermore, when TSWV was acquired by larvae, the virus was transtadially passed and persisted through adulthood. From these data we conclude that TSWV ingested by adult WFT is apparently degraded or altered in the midgut lumen and/or epithelial cells, such that events leading to dissemination to the hemocoel and secondary target organs in the insect cannot occur. Consequently, these adult WFT do not become infective unless virus acquisition feeding occurs during the larval stages. These data represent an important first step toward investigating molecular and biochemical mechanisms governing vector specificity, transmission efficiency, and use of thrips serology to determine the epidemiological significance of thrips populations.

*Additional keywords:* Bunyaviridae, epidemiology, immunocytochemistry, Tospoviruses.

Tomato spotted wilt virus (TSWV) is transmitted by several species of thrips (order: Thysanoptera) and seriously affects production of food, fiber, and ornamental crops worldwide (3,15,26,35–37,40–43). The thrips-TSWV relationship is unusual among insect-transmitted plant viruses in that adult thrips can only transmit TSWV when acquisition occurs during larval feeding on TSWV-infected plants (22,26,41,42). Once acquired by thrips larvae, TSWV is circulative in the insect and is passed transtadially (40–42). Viral replication may occur in thrips cells (9,16,48,50), and the virus is transmitted persistently by adult thrips (40–42). In contrast, adult thrips that have not fed on infected plants during larval instars cannot become viruliferous even if they are allowed lengthy feeding on TSWV-infected plants (37,40–43). Although this unusual insect-virus relationship has been recognized for nearly 60 yr, until recently the underlying mechanisms have eluded entomologists and plant pathologists alike (2,3,11, 27,35,38,40–42,46).

Morphological and physiological explanations for this phenomenon, such as the adult midgut preventing passage of the virus to the insect hemocoel or differences in larval and adult midgut pH affecting virus stability or dissemination, have been considered (11,41). Recently, differences in the feeding behavior of larval and adult thrips were considered as a potential mechanism underlying the differential ability of the two stadia to acquire and transmit TSWV (50). Technical limitations coupled with the minute size of this insect (0.5 × 1.5 mm for an average adult female) limited early research efforts to an organismal and descriptive level.

In this article, we elucidate a midgut barrier preventing acquisition of TSWV by adult western flower thrips (WFT), *Frankliniella occidentalis* (Pergande). Furthermore, we define the fate of TSWV in adults given acquisition feeding on infected plants as adults and not as larvae. Finally, we propose a cellular explanation regarding the inability of adult thrips to acquire TSWV. Implications of our findings to epidemiological studies of TSWV occurrence and spread are also discussed.

## MATERIALS AND METHODS

**Plant material.** *Emilia sonchifolia* (L.) DC. ex Wight and *Datura stramonium* L. were grown from seed in greenhouses at the University of Hawaii-Manoa and used for maintenance of virus isolates and thrips acquisition studies. A severe TSWV isolate (L strain) collected from tomato on the Hawaiian island of Maui was used in all experiments. The TSWV isolate was maintained by thrips transmission in *E. sonchifolia*. Plants to be used for thrips acquisition in all experiments were created by sap-inoculating the first true leaves of *D. stramonium* seedlings with thrips infected *E. sonchifolia* tissue. Techniques used for sap inoculation have been previously described (8,10,18). Plants with symptoms of TSWV were routinely tested with enzyme-linked immunosor-

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bent assay (ELISA) as previously described (18) to verify TSWV infection.

**Test insects.** The WFT was used in all experiments to maintain the TSWV isolate used as an acquisition source in these investigations. Insects were reared and maintained in the laboratory on green bean pods, *Phaseolus vulgaris* L. 'Green Crop,' placed in 227-g plastic deli cups with snap-on lids (Kaiser Aluminum and Chemical Corp., Pleasanton, CA). A 7.50-cm hole, covered with 178-mesh monoester silk screen (NBC Industries Co., Tokyo, Japan) in the lid, allowed ventilation but prevented escape of the thrips. The virus-free status of colony-reared WFT was verified by periodic ELISA testing of adult thrips randomly sampled from the colony using previously described techniques (8).

**Virus transmission experiments.** The differential ability of larval and adult WFT to acquire and transmit TSWV was tested by allowing late first-instar larvae and 1-day-old adults from the virus-free colony to feed for 3 days on excised leaves of TSWV-infected *D. stramonium*. Larvae from these cohorts were then placed on green bean pods and allowed to pupate and complete their development to the adult stage. Ten-day-old adults from larval acquisitions were placed in groups of 50–100 on each of eight to nine healthy *E. sonchifolia* for a 3-day inoculation access period. Insects from adult acquisitions were maintained on noninfected green bean pods for 10 days postacquisition to ensure that adults from both adult and larval acquisitions had a similar latent period. Adults from adult acquisitions were then placed on 23 healthy *E. sonchifolia* test plants as described above. In addition, a control was used in which larval and adult thrips were fed on healthy *D. stramonium,* held on green bean pods, and placed on healthy *E. sonchifolia* test plants. Subsamples of WFT larvae and adults from TSWV treatments and controls were taken immediately after acquisition feeding and just before the inoculation access period on healthy test plants for ELISA testing (six to 10 insects per treatment per sample date × two sample dates per treatment × two treatments). All test plants were then held in the greenhouse and observed for TSWV symptoms. Plants were observed for symptoms of TSWV infection and then tested with ELISA as previously mentioned.

**Immunogold labeling and transmission electron microscopy (TEM).** Putative TSWV particles were visualized in WFT cells using TEM to view WFT tissue embedded in Spurr's resin and WFT cells prepared with cryo-immunocytochemical techniques. Larval and adult WFT were prepared for embedding in Spurr's resin and visualization with TEM using previously described techniques (49). Immunocytochemistry was conducted solely on cryologically prepared sections because of loss of viral antigenicity in Spurr's resin-embedded specimens (unpublished data). Preparation of thrips for cryo-immunocytochemistry is described in the following protocol. Individual larval ($n = 2$ fed on infected plants and 2 fed on noninfected plants) and adult ($n = 2$ fed on infected plants and 2 fed on noninfected plants) WFT were placed on a glass slide in a drop of fixative (0.2% glutaraldehyde + 4% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.4). Thrips antennae, wings, and legs were removed within the fixative drop. Thrips were placed in a vial of the same fixative for 2–4 h at room temperature and then refrigerated at 4 C for 4–5 days. Following initial fixation, thrips were cut into three sections (head and partial thorax, thorax and partial abdomen containing midgut, and abdomen containing hindgut). Thrips pieces were then placed in a cryoprotectant consisting of 1.6 M sucrose in 10–30% polyvinylpyrrolidone (PVP-10,000) as described by Tokuyasu (47) and held at 4 C for 2 days. Cryoprotected thrips tissues were mounted on cryo-pins, rapidly frozen in liquid nitrogen, and transferred to the cryo-chamber of a low-temperature sectioning system (Reichert-Jung FC4D-Ultracut-E cryosystem, C. Reichert Optische Werke, Wien, Austria), where they were stored for at least 15 min before sectioning. Thin sections (90 nm thick) of the alimentary canal, made with a glass knife at −100 C, were removed from the knife blade on a wire loop with a drop of 2.3 M sucrose and floated onto a Formvar-coated 150-mesh hexagonal nickel grid at room temperature. Subsequently, the grid was placed on a drop of 0.5% gelatin (Teleosteam/Cold-Water Fish Skin; from Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) (6). Ultrathin frozen sections were labeled with a polyclonal TSWV antibody provided by Dennis Gonsalves, Cornell University (1:25 dilution in 5% fetal calf serum [FCS]-PBS), and tagged with a protein A-gold marker (10 nm) (1:15 dilution in 5% FCS-PBS) from E. Y. Laboratories, Inc. (available from SPI, West Chester, PA). As a measure of nonspecific labeling by protein A-gold, similar sections that were not reacted with any antibody were labeled with the same dilution of this colloidal gold marker.

Positive controls included partially purified TSWV from *D. stramonium* prepared using the methods of Gonsalves and Trujillo (18) and provided by Min Wang, Department of Plant Pathology, University of Hawaii. TEM grids were floated on liquid suspensions of purified TSWV preparations for 10 min followed by a 10-min fixation with 4% glutaraldehyde. Purified particles were reacted with polyclonal antibody against TSWV (1:25 dilution in 5% FCS-PBS) followed by protein A-gold (1:15 dilution in 5% FCS-PBS) and, as a serum control, with polyclonal antibody against papaya ringspot virus (1:25 dilution in 5% FCS-PBS) for 30 min at room temperature followed by protein A-gold (1:15 dilution in 5% FCS-PBS) for 30 min at room temperature. An additional control included grids coated with purified virus particles and reacted with protein A-gold in the absence of antibody. Washes with 10% FCS-PBS were done between each step in this process. Sections and purified virus preparations were viewed on a Zeiss 10C transmission electron microscope (Carl Zeiss, D-7082, Oberkochen, Germany) at an accelerating voltage of 80 kv.

Colloidal gold labeling of thrips tissues was quantified by making photomicrographs of treated tissues (noninfected and infected thrips midguts treated with polyclonal antibody against TSWV followed by protein A-gold or protein A-gold alone) at a magnification of ×20,000. For each treatment, 20 photomicrographs were taken using up to six different sections of two insect midguts on each of three grids. Gold particles were quantified in each photomicrograph by counting particles in three randomly selected 3-cm squares. The mean number of gold particles per square micrometer of tissue per treatment were then compared using a two-way analysis of variance (SAS, version 6, SAS Institute Inc., Cary, NC).

**TSWV acquisition and movement in WFT.** Late first-instar and adult WFT taken directly from laboratory-reared, virus-free colonies were given varying acquisition access time on groups of excised TSWV-infected *D. stramonium* leaves with early symptoms of TSWV (vein clearing and mosaic, 7–14 days postinfection) (treatments = acquisition access times). Infected leaves were selected from five to 10 different TSWV source plants and pooled for each treatment. As a control, laboratory-reared, virus-free insects were treated in the same manner, except feeding access was to noninfected *D. stramonium* leaves. Noninfected leaves also were selected from several plants and pooled for each treatment.

Treatments included thrips larvae and adults feeding on excised leaves for 1, 2, 4, 8, and 24 h, after which they were fed on sucrose under thinly stretched Parafilm (25) in modified Tashiro cages (32) for 1 h before processing. Feeding on sucrose allowed the insects to clear the feeding tube formed by the maxillary stylets and the esophagus of plant material and TSWV particles. These treatments were replicated three times with larval WFT and three times with adult WFT. Because of the time required for processing of insects, one larval and one adult replication of each treatment were performed per day.

Insects from each treatment were divided into three groups. The first was prepared for embedding in Spurr's resin and viewing with TEM using previously described methods (49) (six larvae per treatment per replication and six adults per treatment per replication). The second group was tested with ELISA as virus-positive thrips controls (two to three larvae per treatment per replication × five treatments × three replications = total of 35 larvae; two to three adults per treatment per replication × five treatments × three replications = total of 35 adults). The third group was tested with ELISA as dissected anterior ends (heads)

and dissected posterior ends (tails). Among the third group, two types of dissections were performed to detect virus dissemination in thrips tissues. Type 1: As a control to determine whether virus particles were residual in the stylets or anterior alimentary canal after WFT were fed on sucrose, dissections were made in which the insect head contained a piece of the subesophageal ganglion, the eyes, and the mouthcone (structure housing the stylets). Remaining nervous tissue, complete salivary glands, fat body, alimentary canal, and all other organs were contained in the posterior or tail end of the insect. This control was conducted on 24-h treatments only (two to three larvae per replication × three replications = total of nine larval heads and nine larval tails; three to four adults per replication × three replications = total of 10 adult heads and 10 adult tails). Type 2: Presence of TSWV in the insect hemocoel was determined by making dissections in which the head end included the insect head and prothorax containing the salivary glands, some nervous tissue, and a small piece of fat body. The tail end of the insect then contained the entire alimentary canal, the majority of the fat body, and all other organs (five to 10 larvae per treatment per replication × five treatments × five replications = total of 87 larval heads and 109 larval tails; eight to 10 adults per treatment per replication × five treatments × three replications = total of 48 adult heads and 48 adult tails). Identifications of thrips organs were based on previous light and electron microscopy of WFT morphology (48,49).

Negative controls consisted of larval and adult WFT fed on noninfected *D. stramonium* in the same fashion as described for TSWV-infected treatments above (two to five larvae per treatment per replication × five treatments × three replications = total of 35 larvae; two to five adults per treatment per replication × five treatments × three replications = total of 35 adults). These noninfected control individuals were not dissected before preparation for testing with ELISA. A subsample from the 24-h treatment of this cohort also was prepared for viewing with TEM (one to two larvae per replication × three replications = total of six larvae; two adults per replication × three replications = total of six adults). Testing with ELISA was performed on intact insects and dissected heads and tails using previously reported methods (8,9).

**Retention of TSWV by adult thrips.** Adult thrips from laboratory colonies were allowed to feed on excised leaves of TSWV-infected *D. stramonium* as described earlier for 3 days. Following acquisition feeding, WFT were maintained on noninfected green bean pods. Green bean pods were changed at 2-day intervals through completion of the experiment. Four treatments were included: insects sampled directly from TSWV-infected plants (no postacquisition period) and insects sampled at 2, 4, and 8 days postacquisition. There were 100 adult WFT per treatment in replication 1 × four treatments, and 50 adult WFT per treatment in replication 2 × four treatments = total of 750 adult WFT. The negative control thrips in this experiment were treated in a similar fashion, except feeding access was on healthy *D. stramonium*. These noninfected controls included 10 adult WFT per treatment × four treatments × two replications = total of 80 adult WFT. The thrips were killed by immersion in 95% ethanol, stored in PBS with Tween 20, and tested for TSWV presence with ELISA (9). None of these thrips was prepared for viewing with TEM.

This experiment was repeated with WFT maintained on 5% sucrose in modified Tashiro cages instead of green bean pods after removal from infected plants. Thrips feces were collected from the surface of the sucrose sachet after 24 h of postacquisition feeding. The feces and a subsample of 20 insects were then tested for virus presence with ELISA. After 8 days of sucrose feeding, thrips were again sampled ($n$ = 20) and tested by ELISA for virus presence. Subsamples were prepared for TEM viewing with no postacquisition period (nine insects) and after 1 and 8 days of postacquisition maintenance on sucrose (six insects per treatment × two treatments = total of 12 insects). As a negative control, adult WFT were fed on noninfected *D. stramonium* and sampled for ELISA as described above (five to seven insects per treatment × three treatments = total of 20 insects) and TEM viewing (six insects with no postacquisition treatment, three insects per 1- and 8-day postacquisition treatments = total of 12 insects).

**Statistical analysis.** Comparison of larval and adult acquisition and retention among all treatments in each experiment were made using chi-square analysis. Differences among larval and adult optical density (OD) readings per treatment were compared using analysis of variance with Tukey's studentized range test to examine differences among means. All statistical tests were completed using SAS, version 6 (SAS Institute).

## RESULTS

**Virus transmission experiments.** Adult WFT given TSWV acquisition access as adults and not as larvae could not subsequently transmit TSWV. Even with long acquisition access and 50–100 adults per test plant, no infection occurred in any of the 23 plants treated. In contrast, when WFT were given TSWV acquisition access as larvae, reared to adulthood, and then given inoculation access to test plants, infection occurred in five of the eight plants treated. No TSWV infections occurred in plants fed upon by noninfected control insects (those fed on noninfected plants during the acquisition period).

Results of ELISA testing of individual larvae show that immediately after acquisition from infected plants, TSWV could be detected in 70% of the insects. OD at 405 nm varied greatly among positive larvae, ranging from a minimum of 0.025 to a maximum of 0.262 (mean OD readings = 0.101 ± 0.091). Among members of this cohort reared to adulthood, TSWV could be detected in 20% of the individuals tested before inoculation access on test plants. In contrast to their larval siblings, OD readings did not vary greatly among positive individuals, ranging from a minimum of 0.118 to a maximum of 0.185 (mean OD readings = 0.152 ± 0.047).

When acquisition feeding was given to WFT as adults and not as larvae, TSWV could be detected with ELISA in more than 40% of the insects immediately postacquisition. OD readings among positive individuals varied greatly, ranging from 0.025 to 0.358 (mean OD readings = 0.109 ± 0.165). Unlike WFT

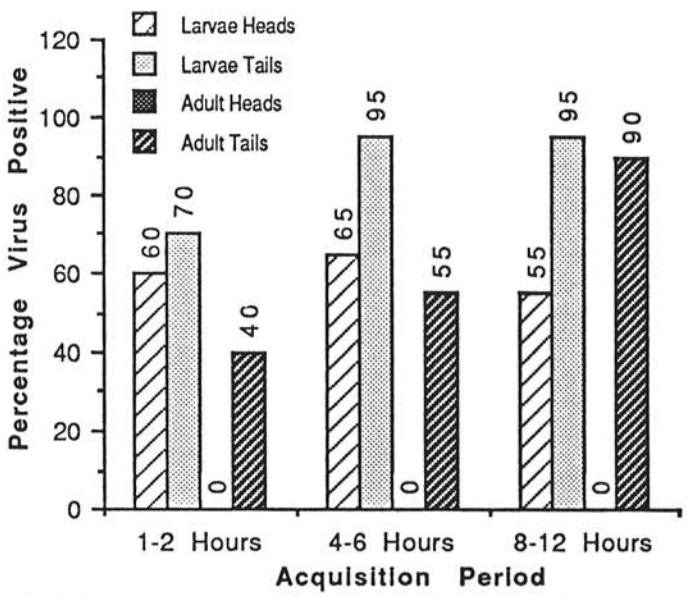



**Fig. 1.** Results of an enzyme-linked immunosorbent assay to detect tomato spotted wilt virus (TSWV) in dissected larval and adult *Frankliniella occidentalis* heads (head and partial thorax of insect containing feeding structures, nervous tissue, salivary glands, and partial fat body) and tails (abdomen and partial thorax of insect containing esophagus, midgut and hindgut, fat body, reproductive organs, etc.) after different acquisition feeding periods on infected plants. The numbers of heads and tails in which TSWV was detected are expressed as a percentage for larvae and adults in each acquisition access period ($n$ = 87 larval heads, 109 larval tails, 48 adult heads, and 48 adult tails).

allowed to acquire TSWV as larvae, virus could not be detected with ELISA in any of these adults when sampled before the first inoculation access on test plants (after 10 days of postacquisition feeding on noninfected plants) (OD readings ranged from −0.003 to 0.003, mean = −0.001 ± 0.001). Virus was not detected with ELISA or TEM observation in control larvae or adults fed on noninfected plants during the acquisition feeding period. None of the adult treatments or noninfected insect controls caused TSWV infection in the more than 23 plants tested.

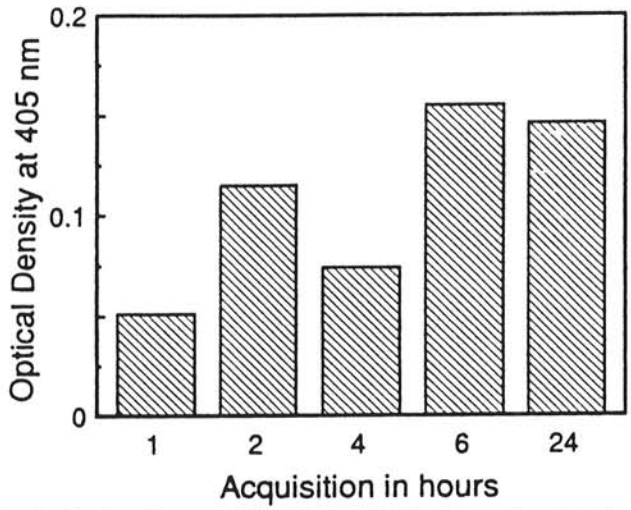
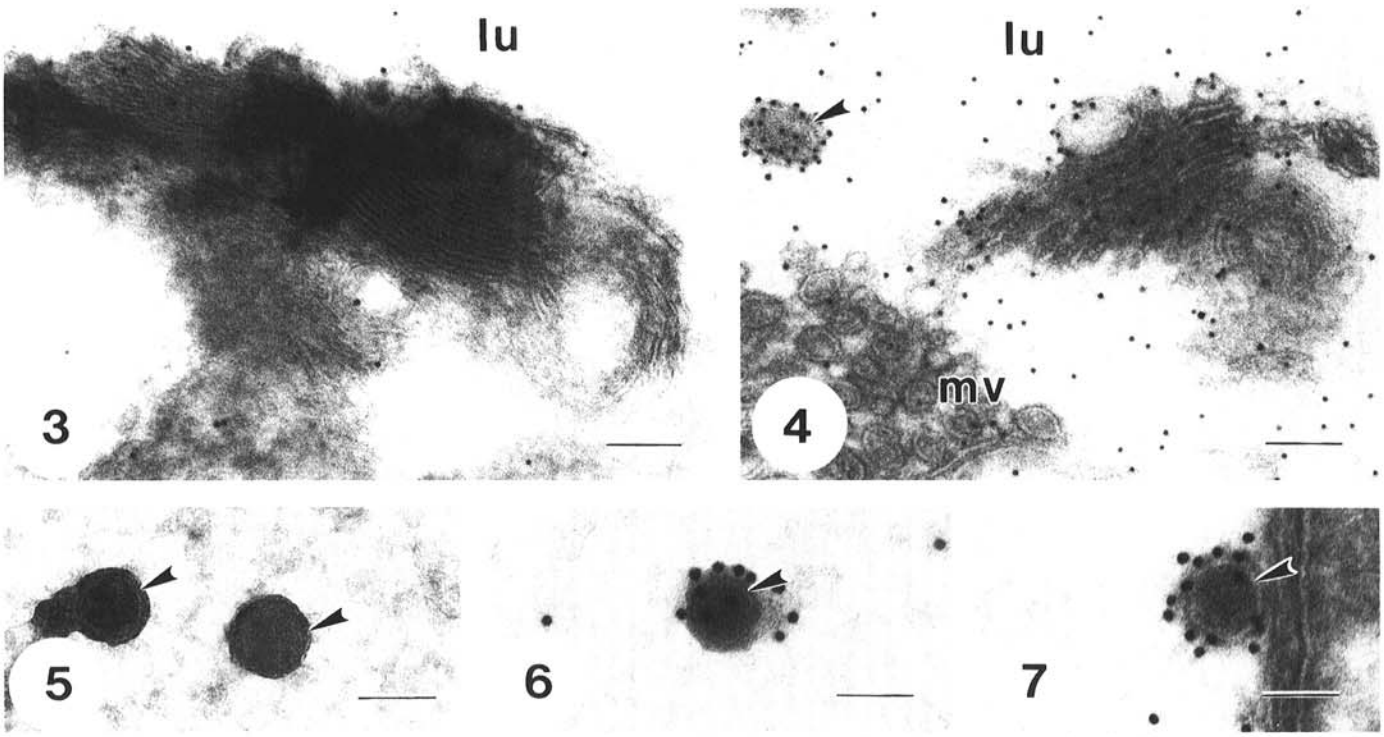


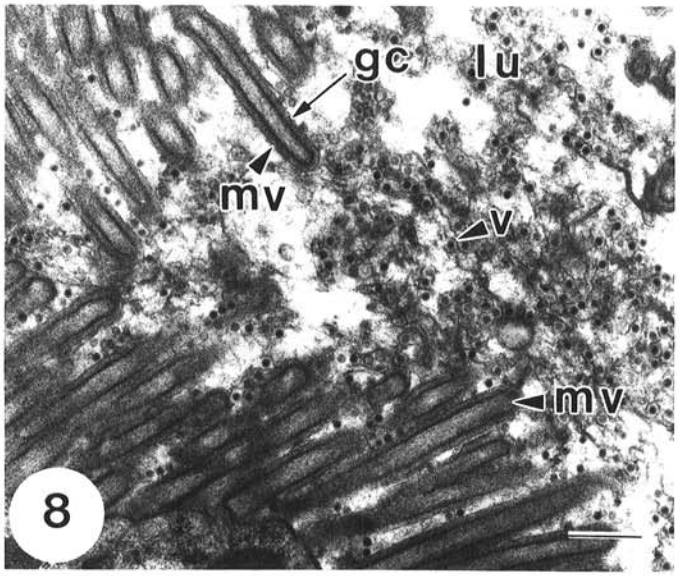

**Fig. 2.** Results of enzyme-linked immunosorbent assay for detection of tomato spotted wilt virus (TSWV) in dissected larval *Frankliniella occidentalis* heads containing salivary glands and partial fat body. Increasing optical density suggests increased TSWV titer in the hemocoel as acquisition feeding on TSWV-infected plants increased ($n = 87$).

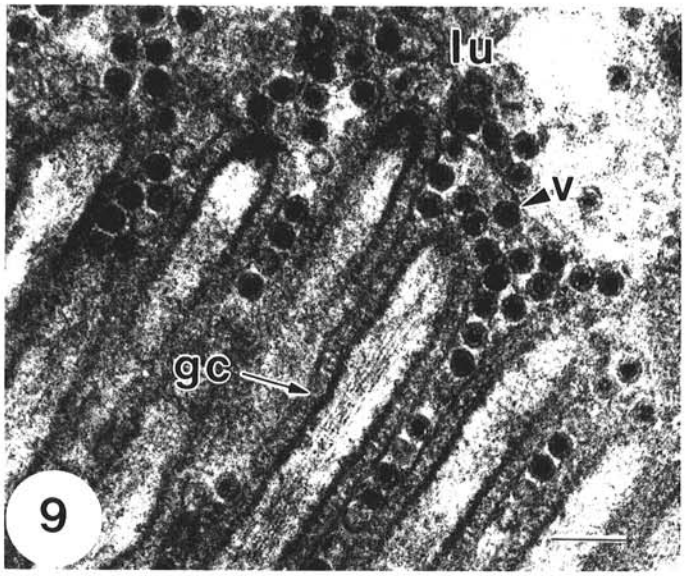
**TSWV acquisition and movement in WFT.** The following sections report results of experiments addressing TSWV uptake and movement in WFT larvae and adults fed on TSWV as adults. Results of ELISA on dissected tail ends (types 1 and 2, containing entire digestive tract) of larvae and adults varied widely. OD readings for positive larvae ranged from 0.025 to 2.663 (mean = 0.588 ± 0.698) and from 0.041 to 1.097 for adults (mean = 0.249 ± 0.209). Among both larvae and adults, increased acquisition time resulted in significantly more positive individuals ($X = 11.447$ and 15.978, $P < 0.003$ and 0.0001 for larvae and adults, respectively). Although virus was detected in more larvae than adults after acquisition feedings up to 6 h (Fig. 1), the differences between the number of positive larvae and adults within each treatment were not significant. As acquisition feeding time increased beyond 6 h, TSWV could be detected in an increasing number of both larvae and adults, again without significant differences between the stadia (Fig. 1). Following the longest virus acquisition period (24 h), TSWV was detected with ELISA in more than 90% of both larvae and adults (Fig. 1).

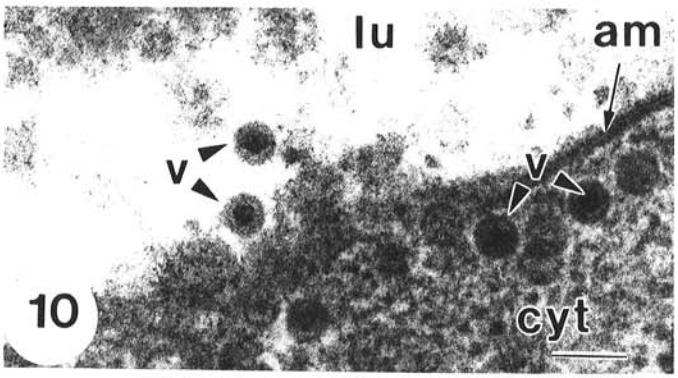
Although both larvae and adults ingested virus during each acquisition treatment, OD readings were significantly higher in larvae than in adults within each treatment ($F = 21.21$, $P < 0.0001$), suggesting that larvae ingested more virus than adults. Virus was not detected using ELISA in larval or adult control insects fed upon noninfected plants with ODs for both stadia ranging from 0 to 0.007 (mean = 0.001 ± 0.0003). Virus particles resembling TSWV were observed with TEM in WFT from TSWV acquisition treatments that were not observed in control insects.

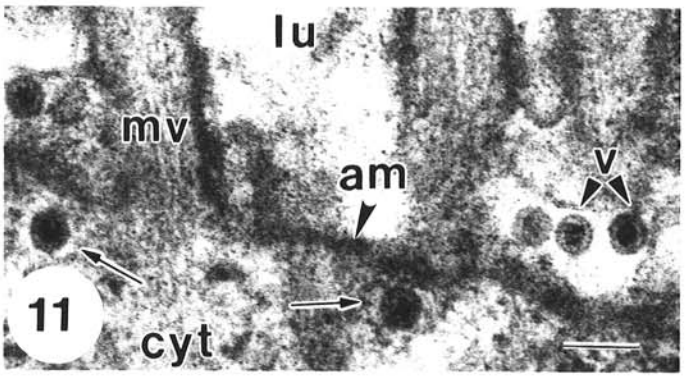


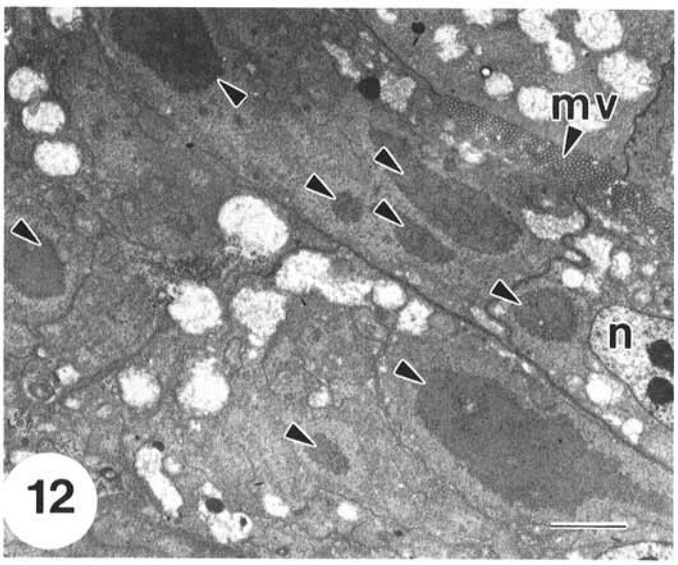

**Figs. 3–7. 3,** Electron micrograph of a thin cryosection of an adult *Frankliniella occidentalis* midgut prepared after feeding on noninfected plant tissues. Section was treated with polyclonal antibody against tomato spotted wilt virus (TSWV) followed by labeling with protein A-gold (10 nm). Membranous material of plant origin is present in the midgut lumen. Very low numbers of colloidal gold particles illustrate the limited nonspecific labeling present in the midgut of thrips fed on noninfected plant tissues. **4,** Electron micrograph of a thin cryosection of the midgut lumen of an adult *F. occidentalis* prepared after feeding on TSWV-infected plant tissues. Section was treated as in **3.** Colloidal gold particles labeling whorl-like membranous material of plant origin and an irregularly shaped, nonenveloped structure can be seen in the midgut lumen. Note cross sections of midgut microvilli did not label with colloidal gold. **5,** Electron micrograph showing the absence of colloidal gold labeling in a serum control. TSWV particles from a liquid suspension of purified TSWV were probed with antibody to papaya ringspot virus followed by protein A-gold (10 nm). Arrowhead indicates viral envelope. **6,** Electron micrograph showing colloidal gold labeling of TSWV particles from a liquid suspension of purified TSWV probed with polyclonal antibody to TSWV followed by labeling with protein A-gold (10 nm). Arrowhead indicates viral envelope. **7,** Electron micrograph of a thin cryosection of a thrips midgut treated with polyclonal antibody to TSWV followed by labeling with protein A-gold (10 nm). Colloidal gold particles can be seen labeling a TSWV particle in the midgut lumen against the apical membrane of a *F. occidentalis* midgut epithelial cell. Arrowhead indicates viral envelope. lu, lumen; mv, microvilli. Bars = 103, 100, 70, 70, and 65 nm for **3–7,** respectively.

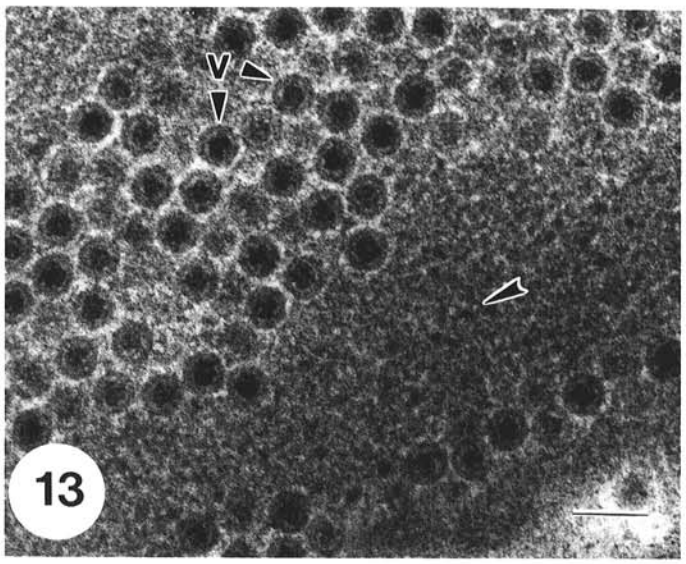



**Figs. 8–13. 8,** Electron micrograph of a thin section of the midgut of an adult *Frankliniella occidentalis* showing tomato spotted wilt virus (TSWV) in the lumen after ingestion from an infected plant. A glycocalyx can be seen associated with the apical plasma membrane of the microvilli lining the columnar epithelial cells. **9,** At a higher magnification the enveloped, spherical characteristics of TSWV particles in the midgut lumen as well as the glycocalyx surrounding the microvilli can be observed. Individual TSWV particles can be seen in close association with the microvillar glycocalyx. **10,** Electron micrograph showing TSWV particles in midgut lumen near the apical membrane and within the cytoplasm of a midgut epithelial cell of an adult *F. occidentalis* after 24 h of feeding on TSWV-infected leaves. **11,** Electron micrograph showing TSWV particles in the midgut lumen and cytoplasm of a midgut epithelial cell of an adult *F. occidentalis* after 24 h of feeding on TSWV-infected leaves. TSWV particles are apparently engulfed at the base of the microvilli and can be seen within smooth vesicles (indicated by arrowheads) near the apical membrane in the cytoplasm of the cell. **12,** Electron micrograph showing TSWV particles accumulating in amorphous, electron-dense masses (arrowheads) in the cytoplasm of many midgut columnar epithelial cells of an adult *F. occidentalis* after 24 h of feeding on TSWV-infected leaves. Cross sections of microvilli lining the midgut lumen can be seen. **13,** Electron micrograph showing TSWV particles in the cytoplasm of a columnar epithelial cell of the midgut of an adult *F. occidentalis* after 24 h of feeding on TSWV-infected leaves. The electron-dense, amorphous material in which they accumulate is also shown (arrowhead). am, apical membrane; cyt, cytoplasm; gc, glycocalyx; lu, lumen; mv, microvilli; n, nucleus; v, TSWV particles. Bars = 621, 207, 140, 140, 3,950, and 140 nm for **8–13,** respectively.

**Virus dissemination from the midgut lumen of larval and adult WFT.** In type 1 dissections, TSWV was not detected with ELISA in larval or adult WFT heads after acquisition feeding on infected plants. Virus could be detected in the tail end of the same individuals, indicating that these insects ingested TSWV and that sucrose feeding cleared the food canal and feeding structures of virus.

In type 2 dissections, even after 24 h of feeding, virus was not detected with ELISA in the heads of adult WFT fed on TSWV-infected plants as adults, although the tail end of the same individuals were virus-positive (Fig. 1). In contrast, among larvae fed on TSWV-infected plants as larvae, TSWV was detected with ELISA in 60% of the type 2 head dissections after only 1 h of feeding on TSWV-infected plants (Fig. 1). The number of larval heads in which TSWV was detected did not significantly increase with increased acquisition time (Fig. 1). Although it was not statistically significant, a trend occurred toward increased OD readings from larval heads as feeding access increased on TSWV-infected plants (Fig. 2).

**Virus uptake and movement in WFT: Cryo-immunocytochemistry and TEM observations.** Cryo-immunocytochemistry and TEM observation of Spurr's resin-embedded WFT adults after feeding on noninfected and TSWV-infected plants revealed whorl-like membranous material of apparent plant origin in the gut lumen of all thrips sampled (Figs. 3 and 4). When feeding occurred on TSWV-infected plants, this membranous material, with virions ranging from 65 to 85 nm and irregularly shaped, nonenveloped structures, was found heavily labeled with polyclonal antisera (Figs. 4 and 7). Some labeling was also observed scattered across the gut lumen of insects fed upon infected plants (Fig. 4), but it was not present in the midgut lumen of insects fed on noninfected tissue (Fig. 3).

The strong immunogold labeling of purified TSWV particles reacted with antibody against TSWV compared with the lack of immunogold labeling on purified TSWV particles reacted with antibody against papaya ringspot virus (serum controls) highlights the specificity of the latter antibody against TSWV antigens (Figs. 5 and 6). Degree and specificity of labeling is further reflected by significant differences in the mean number of colloidal gold particles present per square micrometer of noninfected and infected thrips midgut tissue labeled with anti-TSWV antibody ($16.22 \pm 0.77$ and $174.17 \pm 8.25$, respectively; $P < 0.0001$). Furthermore, nonspecific labeling by protein A-gold alone was minimal, as indicated by significantly fewer colloidal gold particles per square micrometer in infected and noninfected tissues labeled in the absence of antibody ($1.90 \pm 0.18$ and $3.88 \pm 0.27$, respectively; $P < 0.0001$).

Among Spurr's resin-embedded adult WFT fed as adults on infected plants and sampled immediately after acquisition (no postaquisition period), varying numbers of viruslike particles could be observed in the midgut lumen of seven out of 15 insects (Figs. 8 and 9). Three of the seven WFT adults with viruslike particles in their midgut lumen also had viruslike particles near and against the lumen and cytoplasm side of the apical plasma membrane of the microvilli (Figs. 8–11). In these three insects, virus particles were observed at the base of the microvilli (Fig. 11), within the cell near the apical plasma membrane (Figs. 10 and 11), and throughout the cytoplasm of many columnar epithelial cells of the midgut (Figs. 7, 10–13).

Within the cytoplasm, many virions were associated with amorphous, electron-dense material (Figs. 10–13). Frequently, virions were observed accumulating in masses of this material throughout the cytoplasm (Figs. 12–15). Often the viral envelope could not be resolved on these virus particles, nor could membranes be seen surrounding these masses of virus and electron-dense material. In some cases, virions in electron-dense masses were associated with noncontiguous arrays of rough endoplasmic reticulum (Fig. 14). In addition, multivesicular bodies were observed within amorphous masses apparently incorporating accumulating virus particles (Fig. 15). Electron microscopy of adult WFT fixed after feeding from noninfected plants did not reveal virus particles, structures resembling virus particles, or virions accumulating in amorphous electron-dense masses (zero out of 15 insects sectioned from acquisition and retention experiments).

As noted above, incorporation of viruslike particles into the midgut epithelia did not occur in every adult ingesting virus as an adult. Even among insects in which incorporation occurred, many TSWV particles ingested were not incorporated into midgut epithelial cells but excreted as demonstrated by the abundance of virus particles present in the lumen of the hindgut of these

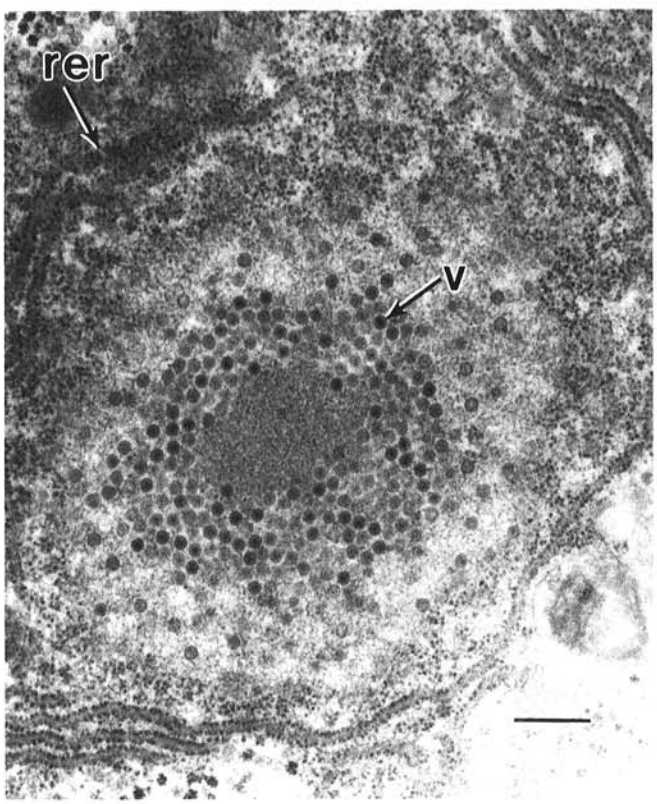



**Fig. 14.** Electron micrograph showing tomato spotted wilt virus (TSWV) particles accumulating in amorphous, electron-dense masses in the cytoplasm of a columnar epithelial cell of the midgut of an adult *Frankliniella occidentalis* after 24 h of feeding on TSWV-infected leaves. Overlapping but noncontiguous arrays of rough endoplasmic reticulum are sometimes seen encircling virus particles within these masses. rer, rough endoplasmic reticulum; v, TSWV particles. Bar = 300 nm.

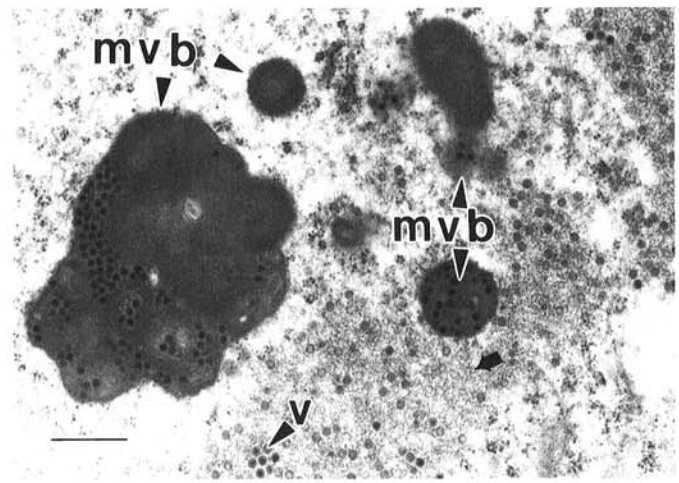
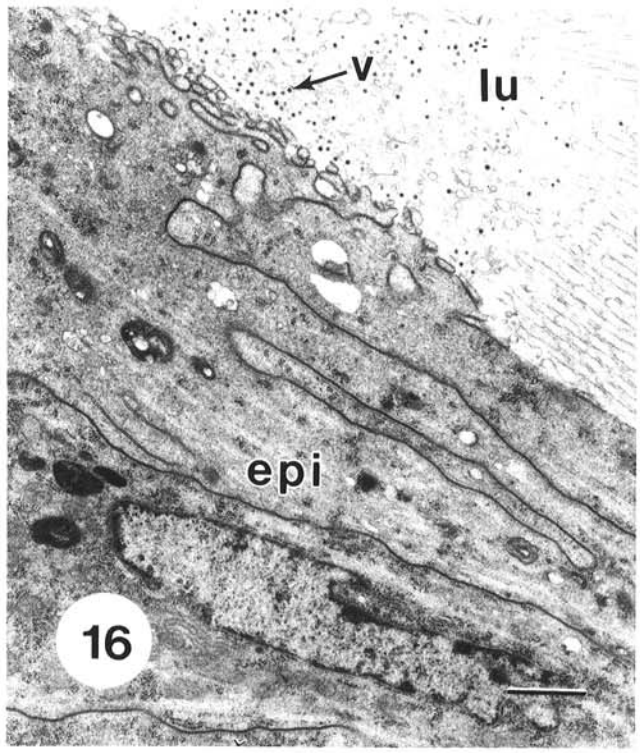
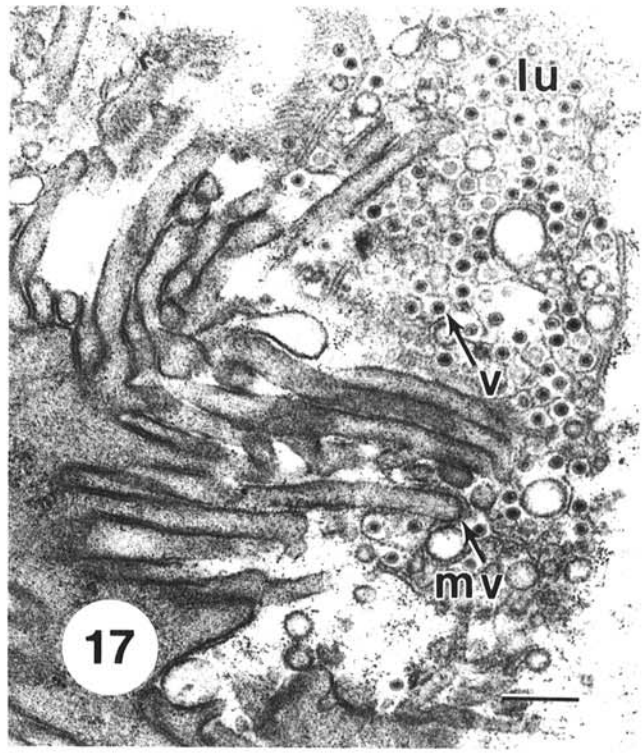



**Fig. 15.** Electron micrograph showing tomato spotted wilt virus (TSWV) particles in an amorphous, electron-dense mass in the cytoplasm of a columnar epithelial cell of the midgut of an adult *Frankliniella occidentalis* after 24 h of feeding on TSWV-infected leaves. Virus particles can sometimes be seen within multivesicular bodies within these amorphous masses. mvb, multivesicular bodies; v, TSWV particles. Bar = 621 nm.




**Figs. 16** and **17. 16,** Electron micrograph of a thin section of the hindgut of an adult *Frankliniella occidentalis.* Tomato spotted wilt virus (TSWV) particles were never observed in the columnar epithelial cells, although many virus particles are present in the hindgut lumen. **17,** At a higher magnification, many TSWV particles can be seen in the hindgut lumen. TSWV particles were not observed crossing the hindgut microvilli and could not be detected within the epithelial cells of the hindgut. The large number of particles found in the hindgut lumen suggest that much of the virus ingested is excreted. epi, epithelial cells; lu, lumen; mv, microvilli; v, TSWV particles. Bars = 751 and 300 nm for **16** and **17,** respectively.

individuals (Figs. 16 and 17). The hypothesis that much ingested TSWV is excreted is further supported by detection of TSWV in adult WFT feces with ELISA (OD = 1.30). Although large numbers of viruslike particles were observed in the hindgut lumen (Figs. 16 and 17), particles were not observed near or against the lumen or cytoplasm side of the apical plasma membrane of hindgut microvilli, nor were virus particles observed within the cytoplasm of hindgut epithelial cells (Figs. 16 and 17). Viruslike particles were not observed in the hemolymph or secondary target organs such as the fat body, nerve cells, or salivary glands in WFT that fed on TSWV-infected plants as adults (zero out of 15 insects sectioned).

In contrast, viruslike particles were observed in the midgut epithelium and hemocoel (data not shown) of two out of six larvae examined with TEM after 24 h of feeding on TSWV-infected plants. In these insects, viruslike particles in the midgut epithelium sometimes accumulated in amorphous, electron-dense masses and were occasionally associated with rough endoplasmic reticulum and multivesicular bodies. Virions in the midgut lumen, epithelial cells, and hemocoel also ranged from 65 to 85 nm in diameter.

**Virus retention by adult WFT.** More than 85% of WFT adults from treatments with no postacquisition period were positive for TSWV presence with ELISA (Fig. 18). After 1, 2, and 4 days of postacquisition feeding on noninfected plants, the number of WFT adults in which TSWV could be detected with ELISA decreased dramatically, with less than 11% of the insects testing positively for virus presence. Within 8 days of postacquisition feeding on noninfected plants, TSWV could be detected in less than 4% of the insects. None of the insects from this experiment was prepared for TEM observation.

When this experiment was repeated and insects were prepared for viewing with TEM, viruslike particles similar to those shown in Figures 8–15 were visualized with TEM in the digestive tract of five out of nine adults subsampled immediately after removal from TSWV-infected plants (no postacquisition period). Among these five individuals, one also had virus particles present in amorphous, electron-dense masses within midgut epithelial cells. After 1 and 8 days of postacquisition feeding on noninfected tissue (Fig. 18), virus particles could not be seen with TEM in the digestive tract or midgut epithelial cells of any of the 12 insects sectioned. Nor were virus particles observed in control insects fed on noninfected plants (zero out of three insects sectioned).

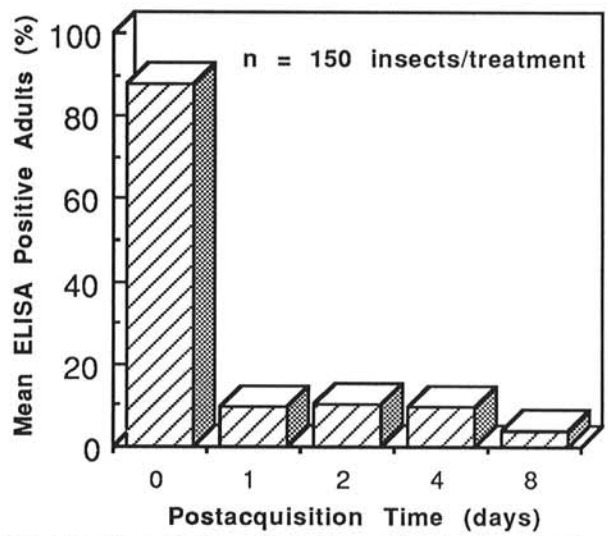



**Fig. 18.** Graphic illustration of retention of tomato spotted wilt virus (TSWV) by adult *Frankliniella occidentalis* after removal from TSWV-infected plants. The percentage of TSWV-positive individuals detected with enzyme-linked immunosorbent assay is shown at increasing intervals of postacquisition feeding on noninfected green bean pods.

## DISCUSSION

Our data clearly show that adult WFT, like other thrips vector species (8,11,22,40–42), must acquire TSWV by feeding during

larval stages to become infective as adults. Clearly, this differential ability to acquire TSWV is not governed by differences in feeding behavior, as data from ELISA and TEM observations show that both larval and adult thrips efficiently ingest TSWV (Figs. 1–9). Serological data (Fig. 1) and TEM observations (Figs. 4–15) indicate that when adult WFT feed on TSWV-infected plants, virions may enter the midgut and be excreted or enter midgut epithelial cells. From studies among adult WFT given acquisition feeding as adults and not as larvae, our evidence indicates that dissemination beyond these cells cannot occur. These data strongly suggest that the midgut and/or midgut epithelial cells of adult WFT act as a barrier to events resulting in virus dissemination to the hemocoel. Serological and ultrastructural evidence, including accumulation of viruslike particles in amorphous, electron-dense masses and in multivesicular bodies, suggests that TSWV entering the adult midgut may be degraded (Figs. 12–15, 18).

In contrast, when WFT larvae ingest TSWV from infected plants, serological and ultrastructural evidence suggests that virions enter the midgut epithelial cells and are ultimately disseminated beyond these cells into surrounding hemocoel. Furthermore, serological evidence shows that TSWV ingested by WFT larvae is transtadially passed and persists in adults developing from these larvae. These conclusions, and the hypothesis that the viruslike particles we observe are TSWV, are supported by several types of evidence including ELISA results, immunocytochemistry, and TEM observations from Spurr's resin-embedded insects.

**Evidence that viruslike particles observed are TSWV.** First, viruslike particles were only observed in insects subsampled from cohorts fed on TSWV-infected plants. Viruslike particles were not observed in a similar number of control insects fed on non-infected plants. If the viruslike particles we observed were another virus, such as an insect virus, we would expect to find viruslike particles in control insects as well as in insects fed on TSWV-infected plants. Secondly, when viruslike particles were seen in adult insects fed on TSWV-infected plants as adults, viruslike particles were only observed in treatments with no postacquisition period. No virus particles were seen in insects sampled after 1 and 8 days of postacquisition feeding on noninfected plants. If the viruslike particles observed in treatments with no postacquisition period represented an insect virus, we would expect to continue seeing virus particles in each of the postacquisition treatments. Finally, cohorts fed on noninfected plants were negative for TSWV presence with ELISA, and cohorts fed on infected plants were positive for TSWV presence with ELISA. These ELISA results verify that insects fed on infected plants had ingested TSWV, making it highly likely that viruslike particles observed in their midgut are TSWV and not any other virus.

**Immunogold labeling.** Cryo-immunocytochemistry on WFT fed on similar TSWV sources at a later time was conducted to provide additional evidence that viruslike particles we observe in WFT larvae and adults are TSWV. Loss of viral antigenicity when insects were prepared for TEM observation by embedding in Spurr's resin prevented successful immunogold labeling of sections shown in Figures 8–17. Specificity of our immunogold labeling in cryologically prepared specimens is well supported by low numbers of gold particles in healthy (Fig. 3) and serum controls (Fig. 5) and by significantly higher numbers of gold particles per square micrometer present on purified virus (Fig. 6) and in sections from infected thrips (Figs. 4 and 7). Furthermore, TSWV was not detected with ELISA in healthy control insects or observed with TEM in control insects fed on healthy plants.

The labeling of TSWV particles, plant material, and irregularly shaped, nonenveloped structures with polyclonal antibody to TSWV in the lumen of the thrips midgut (Fig. 4) resembles that observed in crude extracts from infected lettuce using similar polyclonal antibody from the laboratory of Dennis Gonsalves, Cornell University (1). It has been suggested from labeling of crude plant extracts with monoclonal antibodies that irregularly shaped, nonenveloped structures represent nonenveloped virus particles that are possibly degrading and exposing nucleoproteins (1), thus revealing epitopes not accessible on fully assembled virus particles.

As a digestive organ, the thrips gut contains many enzymes (7,49) that could contribute to degradation of ingested virions. In addition, thrips feed by ingesting the cytoplasm from individual cells (7). In a plant cell where TSWV is replicating, many non-assembled virus proteins are present (24). Thus, the heavy labeling scattered in the lumen of the thrips midgut after feeding on TSWV-infected tissues (Fig. 4) may represent viral proteins released from degrading virions or nonassembled viral antigens ingested from the infected plant. Although this scattered labeling is not on recognizable structures, the immunolabeling is clearly specific to viral antigens because no such labeling occurred in the midgut lumen of WFT fed on noninfected plant material (Fig. 3). Surface labeling of assembled virions (Figs. 6 and 7) suggest that only epitopes on the viral membrane glycoproteins are exposed to the antibody when the viral envelope is intact.

Several histological studies of infected plants report TSWV particles closely associated with endoplasmic reticulum and suggest that replication and virus packaging may occur in the vicinity of the endoplasmic reticulum (5,23,24). Heavy labeling of membranous material in the gut lumen of thrips fed on infected plants (Fig. 4) may result because TSWV replicative processes may be occurring in membranous structures in the plant material on which the insects were feeding. The membranous material we observe is clearly of plant origin, as it was observed in the midgut lumen of insects fed on both noninfected and infected plants. In addition, observation of Spurr's resin-embedded insects with TEM shows that virions are associated with membranous material throughout the digestive tract (Figs. 8, 16, and 17).

**Virus movement in thrips cells.** Results from ELISA of dissected larval heads containing the salivary glands and partial fat body indicate that TSWV moves beyond the epithelial cells of the alimentary canal during larval acquisition because 1) none of the digestive tract is present to contaminate these samples, and 2) all larval and adult dissections containing the feeding structures only were ELISA-negative, indicating that sucrose feeding before dissection successfully prevented virus contamination of the feeding structures.

Increasing virus titers in larval heads that contain the salivary glands and a piece of the fat body (indicated by increased OD readings) suggest that TSWV movement from the larval midgut to the hemocoel occurs after a short time and may be cumulative as virus acquisition feeding time increases (Fig. 2). It seems likely that as insects ingest more TSWV, more virions cross the epithelial cells to the hemocoel, where they become available for entry to cells and organs, including the salivary gland from which plant infection likely occurs. Although no studies specifically link infection of thrips salivary glands to transmission of TSWV, considerable evidence supports the hypothesis that this is the case. For example, larval acquisition of TSWV results in transtadial passage (9), and virus particles are present in the fat body and brain of larvae and in the fat body, brain, and salivary glands of adults developing from cohorts developing after larval acquisition of TSWV (9,48,50). The amount of virus ingested and crossing the apical plasmalemma of larval thrips is probably important in determining thrips transmission efficiency. This idea is supported by previous studies that suggest that increases in TSWV titers ingested by larvae may result in increased transtadial passage (a higher percentage of viruliferous adults) (9,50).

In contrast to results regarding larval acquisition, TSWV was not detected with ELISA in the head end of any adult WFT given acquisition feeding as an adult or observed with TEM beyond the alimentary canal lumen or midgut epithelial cells after acquisition feeding on infected plants (Figs. 1 and 8–17). Furthermore, plant virus inclusion bodies, dense masses (sensu Urban et al [51]), or viroplasms resulting from TSWV replication, such as those observed in plants (4,5,23,24,33,51) and in adult thrips given acquisition feeding during larval stages (48,50), were not observed with TEM in the midgut epithelial cells of these adult WFT. Hence, virus replicative processes that apparently occur in adult thrips when they acquire virus as larvae apparently cannot occur when virus acquisition feeding is in the adult stage.

**Fate of TSWV in adult WFT given acquisition feeding as adults.** Evidence that TSWV incorporated into midgut epithelial cells is retained and degraded is supported by TEM observations in adults sampled after no postacquisition period and after 1 and 8 days of postacquisition. Among these treatments, TSWV particles were observed only in the midgut lumen and epithelia of adult WFT prepared for observation without a postacquisition period (Figs. 5–15). Virus particles were not seen in WFT adults prepared for observation after 1 or 8 days of postacquisition feeding on noninfected substrate. These results were further supported by ELISA data indicating that the number of WFT adults in which TSWV could be detected decreased dramatically after 1 and 2 days of postacquisition feeding on noninfected plant material and were detected in less than 4% of the individuals tested after 8 days of postacquisition feeding (Fig. 18). The polyclonal antibody used for thrips ELISA detects all TSWV structural proteins. Thus, detection of viral antigens with ELISA in thrips sampled after 1 day or more of postacquisition feeding likely represents presence of degrading viral antigens, because assembled virions could not be seen with TEM.

Ultrastructural evidence provides further support for the hypothesis that TSWV degrades in adult WFT given acquisition feeding as adults. Virions in the epithelial cells of these adult WFT were frequently observed accumulating in masses within amorphous, electron-dense material (Figs. 5–9). These masses resemble amorphous inclusion bodies described in insect and plant cells infected with a variety of viruses (30,39). Although inclusion bodies are common to the cytopathology of many virus-infected plant and animal tissues, little is known regarding specific functions of these structures (30,39). Recent ultrastructural and serological evidence indicates that some plant viral inclusions may be associated with essentially inert collections of viral components or result from a variety of virus-cell interactions, including replication or degradation (30,39). The composition and function of the masses we observe are as yet unknown. Their ultrastructural resemblance to inclusions observed in plant cells infected with cauliflower mosaic virus thought to serve as cellular centers for virus protein degradation (39) suggests an association with degradative processes. Furthermore, presence of multivesicular bodies incorporating virus particles within some of the amorphous, electron-dense masses (Fig. 15) lends support to the hypothesis that an autophagic and/or degradative process is occurring. Further studies will be required to fully resolve the composition and role of these masses in thrips cells.

**Epidemiological implications.** The knowledge that adult thrips ingest and retain TSWV in midgut epithelial cells (Figs. 2–11 and 13) has important implications relative to epidemiological studies using ELISA to assay adult thrips collected directly from TSWV-infected crop plantings. Unless measures are taken to eliminate adults that just fed on infected plants, studies of this nature will tend to overestimate numbers of viruliferous thrips. Our data regarding adult retention of TSWV after removal from infected leaves (Fig. 13) suggest that ELISA could be used in developing thrips infectivity indices and in estimating numbers of infective thrips by dissecting thrips before testing or by holding live thrips on virus-free green bean pods for at least 5 days before testing. Although these techniques may complicate epidemiological surveys, accurate quantification of potentially viruliferous thrips present near susceptible crop plantings could be achieved.

**Possible cellular explanations for a midgut barrier to TSWV acquisition by adult WFT.** Our findings indicate that the midgut and midgut epithelial cells of WFT adults act as a barrier to virus acquisition, limiting dissemination of virus particles to the insect hemocoel and organs where the virus may replicate (16,49,50) or be transmitted to plants during feeding, such as the salivary glands (50). Thus, when acquisition feeding occurs in the adult stage, thrips are prevented from inoculating plants with TSWV. When acquisition feeding occurs during larval instars, virus particles are readily disseminated (Figs. 1 and 2), likely undergo replicative processes (16,49,50), and can be transmitted to plants. The mechanisms limiting dissemination of TSWV from the midgut and midgut epithelia of adults ingesting TSWV as adults may involve receptors at the midgut basement membrane and/or may be influenced by other conditions, such as pH or enzyme processing, to which the virus is subjected within the midgut lumen and/or in the cytoplasm of epithelial cells (17,21,31). By analogy to other members of the Bunyaviridae (12–14,34), internalization and dissemination of TSWV particles in insect tissues is likely to be greatly influenced by pH-mediated apical and basal membrane fusion, processes leading to adsorptive endocytosis, and enzyme processing of viral envelope glycoproteins (4,13,19,20,28,29,31,44,45). The environment of the vector digestive tract, particularly the presence of proteolytic enzymes under certain conditions, is known to alter the surface characteristics of certain viruses and lead to modification of virus surface proteins governing receptor-mediated events leading to virus internalization (28). For example, exposure of La Crosse virus, a Bunyavirus, to proteolytic enzymes, such as those that may be found in its mosquito vector midgut, increase virus affinity for mosquito cells (28) and may be necessary to expose attachment proteins on the virion surface before attachment to and infection of midgut cells can occur. In light of our findings and the similarities between TSWV and other members of the Bunyaviridae, early hypotheses regarding the role of pH differences between larval and adult thrips warrant reexamination (11) on a cellular level. In addition, the role of midgut proteolytic enzyme activity in differing pH environments that may exist in larval and adult thrips should be investigated as mechanisms underlying the midgut barrier we describe.

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